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Non-destructive imaging of an individual protein

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Abstract

Imaging a single biomolecule at atomic resolution without averaging over different conformations is the ultimate goal in structural biology. We report recordings of a protein at nanometer resolution obtained from one individual ferritin by means of low-energy electron holography. One single protein could be imaged for an extended period of time without any sign of radiation damage. Since the fragile protein shell encloses a robust iron cluster, the holographic reconstructions could also be cross-validated against transmission electron microscopy images of the very same molecule by imaging its iron core.

All conventional methods for investigating the structure of biomolecules such as proteins suffer from significant drawbacks. X-ray crystallography, nuclear magnetic resonance (NMR) and cryo-electron microscopy require averaging over a large number of molecules, usually more than 10^6 similar entities¹ and as a consequence conformational details remain undiscovered. Furthermore, these methods can only be applied to a small subset of biological molecules that either readily crystallize to be used for X-ray studies or are readily soluble and small enough (< 35 kDa) for NMR investigations.

Emerging X-ray coherent diffraction imaging (CDI) projects envision imaging the molecule instantaneously before it is destroyed by radiation damage². Recently, Seibert et al. succeeded in imaging a single highly symmetric 750 nm diameter mimivirus with 32 nm resolution³. While there was the initial belief that this technique would provide high resolution images of a single biomolecule, it is now evident that averaging over a large number of molecules cannot be omitted even with the brightest source of the X-ray free electron laser (X-FEL) projects^{4,5}.

We have recently demonstrated that electrons with kinetic energies in the range of 50-200 eV do not cause radiation damage to biomolecules, enabling the investigation of an individual molecule for an extended period of time⁶. Since the electron wavelength associated with this kinetic energy range is between 0.86 Å (for 200 eV) and 1.7 Å (for 50 eV), low-energy electrons have the potential for non-destructive imaging of single biomolecules^{7,8} and in particular individual proteins at atomic resolution.

Our low-energy electron holography experimental scheme transcribes the original idea of holography invented by Dennis Gabor⁹. A sharp tungsten tip acts as field emitter of a divergent coherent electron beam¹⁰. As illustrated in Figure 1, the sample is brought in the path of the coherent electron wave. The electrons scattered off the object interfere with the unscattered so-called reference wave and at a distant detector the interference pattern is recorded¹¹. A hologram, in contrast to a diffraction pattern, contains the phase information of the object wave and the object can be unambiguously reconstructed¹². The numerical reconstruction of the hologram is essentially achieved by back propagation to the object plane which boils down to evaluating the Kirchhoff-Helmholtz integral transformation.

The protein of interest needs to be free-standing in space when exposed to the low-energy electron beam. Therefore the protein is attached to a carbon nanotube suspended over a hole. Strictly speaking, arrays of small holes of 240 nm in diameter are milled in a carbon coated silicon nitride membrane by means of a focused gallium ion beam. The nanotube-ferritin complex is kept in aqueous solution of which a droplet is applied onto the holey membrane. Once the droplet has dried out some of the nanotubes remain across holes providing free-standing ferritin molecules. These molecules can then be examined in our low-energy electron microscope¹¹.

Prior to the described preparation procedure the carbon nanotubes undergo acid treatment in order to form carboxyl groups on the outer wall and hence disperse efficiently in ultra highly purified water. Adding a buffered solution of proteins, the latter eventually bind to the nanotubes by dipole forces, as schematically illustrated

in the inset of Figure 1. This preparation does not rely on specific features of ferritin and hence is applicable to a large class of biomolecules.

Here we report images of ferritin at nanometer resolution obtained from one individual protein by means of low-energy electron holography. The proteins could be imaged for an extended period of time without any sign of radiation damage. Ferritin is a globular protein with a molecular weight of 450 kDa and is composed of 24 subunits, the outer diameter of the protein amounts to 11-12 nm and the inner cavity exhibits a diameter of 8 nm^{13,14}. Up to 4500 iron atoms can be stored in the cavity and the main function of ferritin in the animal metabolism is the regulation of the iron level in the body¹⁴. Recently, it has been shown that ferritin plays an essential role in the progression of neuronal degenerative diseases like Alzheimer or Parkinson¹⁵. Understanding the function of ferritin in more detail by identifying its various conformations, would be an important step towards a better comprehension of the mechanism behind these diseases.

In Figure 2(a), an example of a low-energy electron hologram of ferritin molecules attached to nanotubes is presented. There are several ferritin molecules chemisorbed onto the nanotubes as evident from the hologram reconstruction. Individual ferritin molecules can be resolved, as shown in Figure 2(b-c). The interference resolution estimated by $\lambda/(2N.A.)$ amounts to approximately 1 nm; here $N.A.=S/(2z)$, where the distance z between source and screen amounts to 68 mm and $S=13$ mm refers to the extent of the hologram on the screen. Note that $N.A.$ is not a constant as in conventional microscopy but refers to the angle under which highest order interference fringes are still detectable. $N.A.$ thus depends on

mechanical stability, ac-magnetic field shielding and the properties (resolution and dynamics) of the detector. The ability to image several molecules exhibiting different orientations and conformations in just one single-shot is a promising feature inherent to our method. The individual ferritin molecules have been monitored for more than 20 minutes without observing any change in the hologram. The corresponding total dose amounts to more than 10^9 electrons/nm² and is at least six orders of magnitude higher than the permissible dose in TEM or X-ray examinations of biomolecules. The globular form and the size are in agreement with the structure proposed by X-ray crystallography and cryo-TEM investigations.

Due to the large amount of iron nuclei stored in the center of the molecule, ferritin can also be detected by high-energy electron imaging techniques¹⁶⁻¹⁹ without any additional heavy metal staining. Though these techniques lead to radiation damage of the protein shell they allow for subsequent cross-validation in terms of presence and location of the molecule.

In Figure 3(a), a hologram of a ferritin sample recorded with electrons of 57 eV kinetic energy is depicted. The presence of a bundle of carbon nanotubes as well as the globular structures on its outer wall can already be identified before the numerical reconstruction has been performed. The result of the latter is presented in Figure 3(b). The right side of the nanotube corresponds to the correct representation, free of twin-image artifacts²⁰, as obtained by single-sideband holography reconstruction²¹. A close-up of the reconstruction of an individual ferritin molecule is displayed in Figure 3(c).

For cross-validation, the sample was subsequently examined in a conventional 80 kV TEM. Figure 3(d) displays the TEM image of the very same sample as shown in

Figure 3(a-c). The 8 nm iron core of the ferritin is apparent at the very same position as in the previous holographic reconstruction. While the position of the ferritin observed in the low-energy point source microscope perfectly corresponds to that observed in the TEM, the way the protein is attached to the nanotube appears to be different. In contrast to the holographic reconstruction where the protein appears as a sphere attached to an elongated structure, the TEM image displays the protein as if it was “melded” with the nanotube. The overall size of the protein observed in the TEM amounts to 9-10 nm. We associate these differences to the radiation damage which has occurred during TEM imaging. Following the high-energy electron investigations, the sample was transferred back to our low-energy electron point source microscope. Now, the damage provoked by the 80 keV kinetic energy electrons is evident if one compares the reconstruction before TEM investigation (Figure 3(e)) with the one after TEM investigation (Figure 3(f)). The way the protein is now attached to the nanotubes coincides with the TEM results. In accordance with single molecule cryo-TEM investigations²² which state that the permissible dose before destroying a protein is of the order of 10 electrons/Å², it is conceivable that our TEM image displays the bare iron core while the protein shell has been decomposed. This is consistent with our observations that the spherical object, presumably just the iron cluster plus amorphous carbon from the decomposed protein shell, appears closer to the supporting carbon nanotube.

Here, we have reported non-destructive investigation of an individual protein by means of low-energy electron holography. The holographic imaging of a single protein is cross-validated by TEM examination. The sample preparation method can be applied to a broad class of molecules.

While the spatial resolution in the holographic reconstructions presented here is already in the nanometer range, it is now an experimental challenge to reach a resolution close to the employed wavelength with an advanced low-energy electron holographic setup.

Besides this engineering challenge of improving interference resolution in holography, another route towards higher resolution is to combine holography with coherent diffraction imaging using low-energy electrons²³. Essential ingredients like a micron sized electron lens²⁴ and the ability to record coherent diffraction patterns²⁵ are already at hand for the pursuit of single molecule structural biology at atomic resolution.

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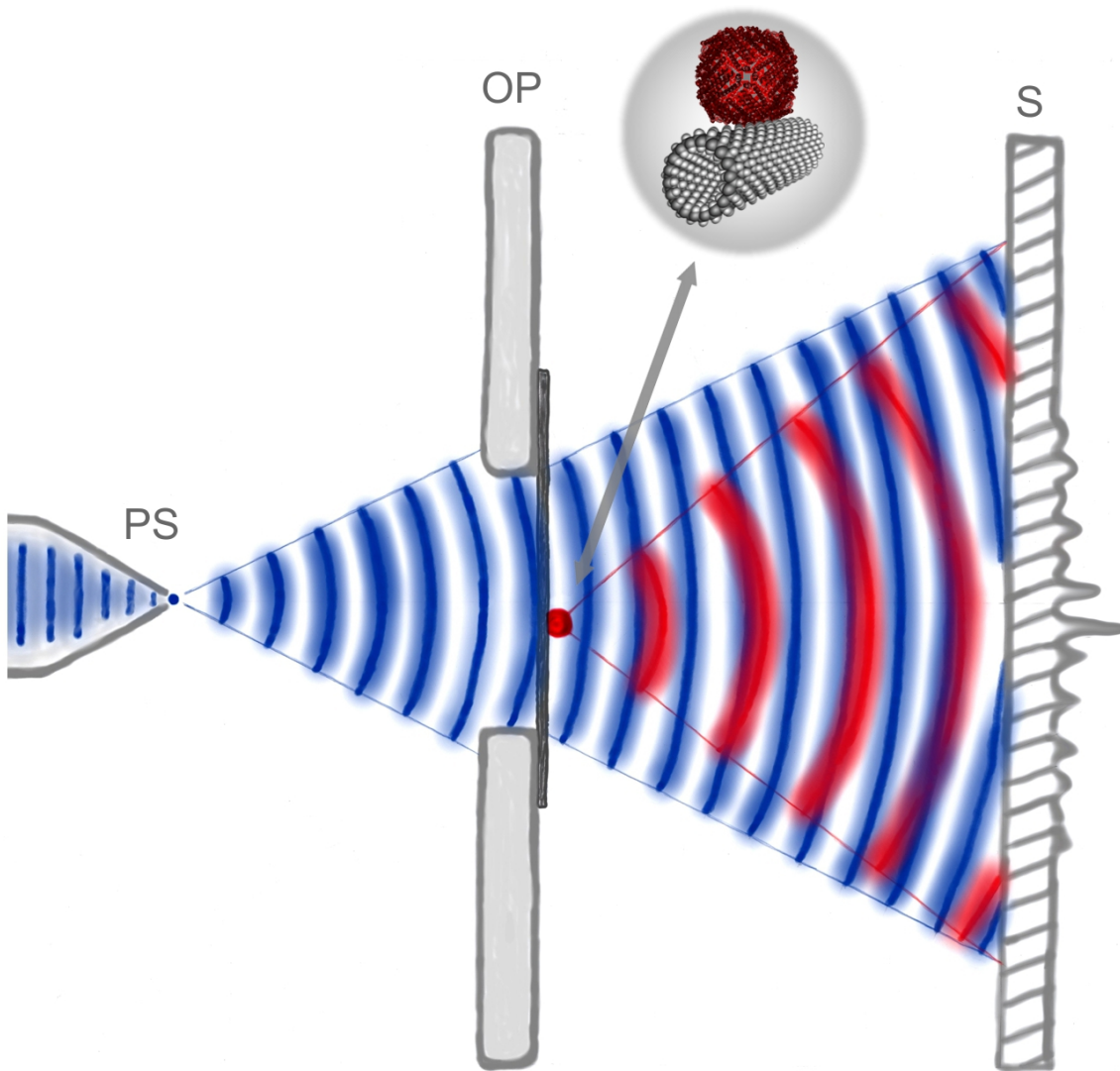


Figure 1: Conduction electrons confined in a pointed W(111) single crystal wire are field emitted into vacuum at an atomic-sized emission area providing a coherent low-energy electron point source (PS). At the less than 1 micron distant object-plane (OP), part of the coherent electron wave is scattered by a ferritin attached to a carbon nanotube constituting the object wave indicated in red. At a 68 mm distant detector screen (S), the far-field interference pattern between object- (red) and reference-wave (blue) - the hologram - is recorded and its digital record is subject to the numerical reconstruction of the protein.

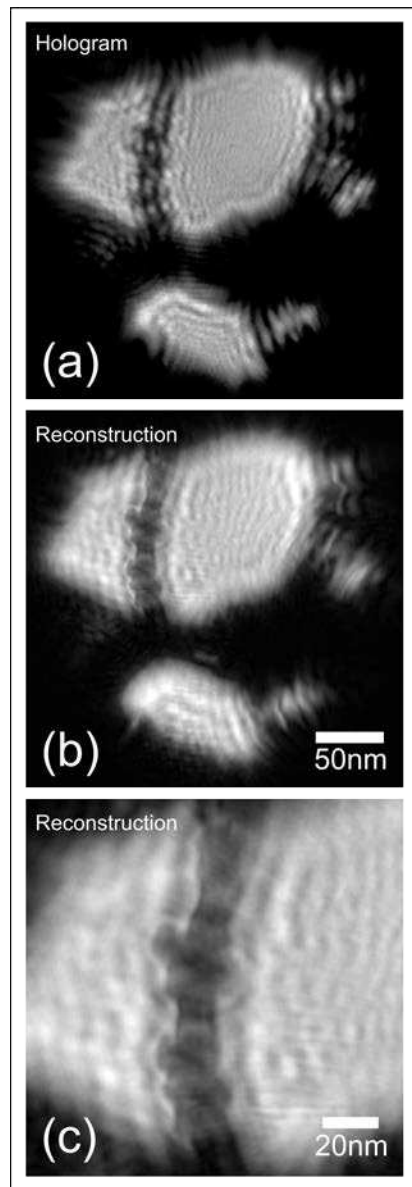


Figure 2: (a) 53eV kinetic energy electron hologram of several ferritin molecules attached to a bundle of carbon nanotubes. (b) reconstruction of (a) obtained at 740 nm distance from the electron source. (c) close-up of (b).

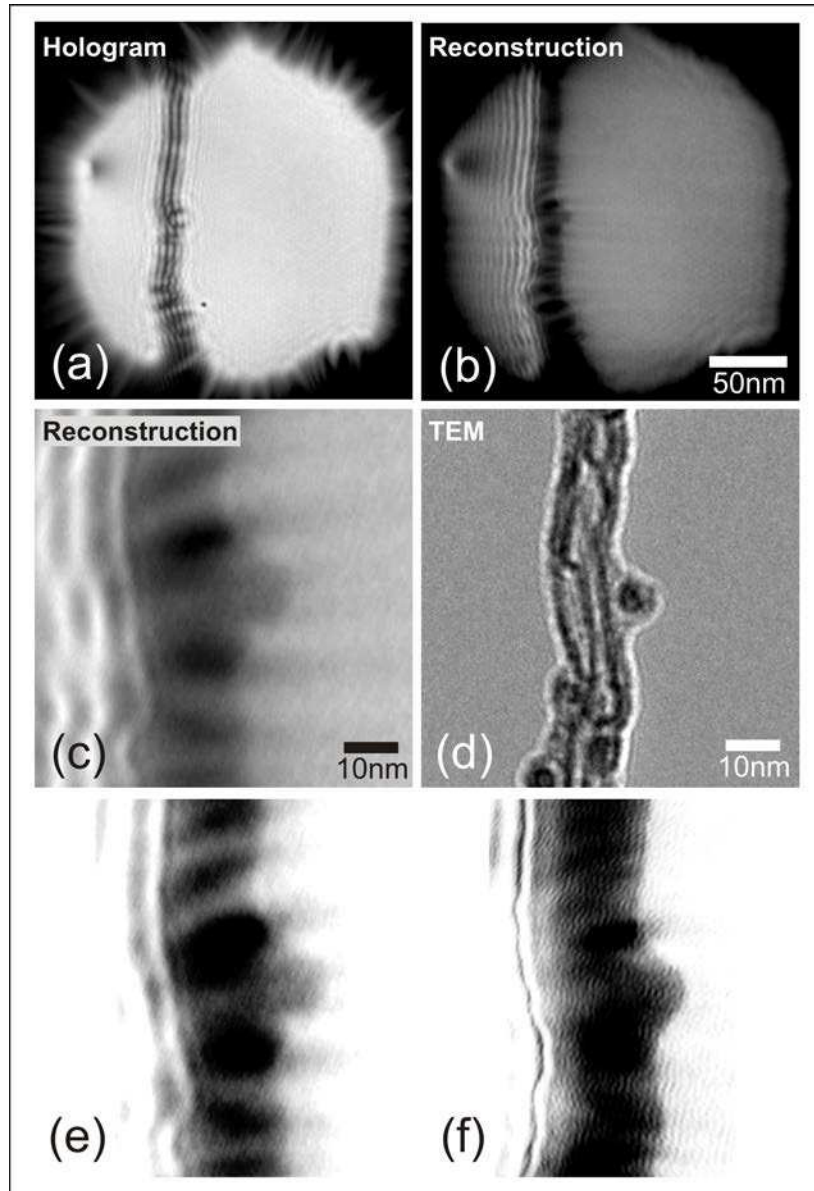


Figure 3: (a) 57eV kinetic energy electron hologram of individual ferritin molecules attached to a bundle of carbon nanotubes. (b) side-band holography reconstruction of (a). (c) close-up of (b). (d) TEM image of the very same ferritin molecule. (e) the same data as the close-up in (c) with changed gamma value to enhance the visibility of the contour of the ferritin. (f) reconstruction of the ferritin hologram recorded after TEM exposure displayed with the same gamma value as in (e).

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